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Regulators of gene expression in Enteric Neural Crest Cells are putative Hirschsprung disease genes

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ABSTRACT

The enteric nervous system (ENS) is required for peristalsis of the gut and is derived from Enteric Neural Crest Cells (ENCCs). During ENS development, the RET receptor tyrosine kinase plays a critical role in the proliferation and survival of ENCCs, their migration along the developing gut, and differentiation into enteric neurons. Mutations in *RET* and its ligand *GDNF* cause Hirschsprung disease (HSCR), a complex genetic disorder in which ENCCs fail to colonize variable lengths of the distal bowel. To identify key regulators of ENCCs and the pathways underlying RET signaling, gene expression profiles of untreated and GDNF-treated ENCCs from E14.5 mouse embryos were generated. ENCCs express genes that are involved in both early and late neuronal development, whereas GDNF treatment induced neuronal maturation. Predicted regulators of gene expression in ENCCs include the known HSCR genes *Ret* and *Sox10*, as well as *Bdnf*, *App* and *Mapk10*. The regulatory overlap and functional interactions between these genes were used to construct a regulatory network that is underlying ENS development and connects to known HSCR genes. In addition, the adenosine receptor A2a (*Adora2a*) and neuropeptide Y receptor Y2 (*Npy2r*) were identified as possible regulators of terminal neuronal differentiation in GDNF-treated ENCCs. The human orthologue of *Npy2r* maps to the HSCR susceptibility locus 4q31.3-q32.3, suggesting a role for NPY2R both in ENS development and in HSCR.

1. Introduction

The enteric nervous system (ENS) of the gastrointestinal tract is composed of neurons and glial cells that are organized in interconnected ganglia in the myenteric and submucosal plexuses. The ENS controls the peristaltic movements of the bowel, fluid exchange between the gut and its lumen, and local blood flow (Furness, 2006; Gershon, 2005). In vertebrates, the ENS is entirely derived from neural crest cells (Le Douarin and Kalcheim, 1999). Vagal (hindbrain) neural crest cells invade the foregut at around E9 in mice (Durbec et al., 1996b), at which point they are referred to as Enteric Neural Crest Cells (ENCCs). The intestinal wall is colonized by ENCCs in a rostral to caudal direction between E9 and E15 in mice and sacral neural crest cells contribute to colonization of the hindgut late during ENS development (Burns and Douarin, 1998; Druckenbrod and Epstein, 2005). A subset of Schwann cell precursors invades the gut postnatally and contributes up to 20% of the neurons in the colon (Uesaka et al., 2015).

Glial cell line Derived Neurotrophic Factor (GDNF) acts as a chemoattractant to migrating vagal ENCCs and directs the migration of ENCCs along the gut (Young et al., 2001). ENCCs express the REarranged during Transfection (*RET*) receptor tyrosine kinase, and RET with its co-receptor GFRα1 is the receptor for GDNF (Durbec et al., 1996a). Mice lacking *Ret*, *Gfrα1* or *Gdnf* fail to colonize the bowel beyond the stomach, leading to intestinal aganglionosis (Enomoto et al., 1998; Moore et al., 1996; Pichel et al., 1996; Sánchez et al., 1996; Schuchardt et al., 1994). Besides its role in migration, RET signaling is important for proliferation of ENCCs

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at E12 and for their survival at E14-E16 in mice (Chalazonitis et al., 1998; Gianino et al., 2003; Taraviras et al., 1999; Uesaka et al., 2008). In order to develop into a mature ENS, post-migratory ENCCs differentiate into various neuronal subtypes and glial cells. GDNF promotes neuronal, but not glial differentiation of ENCCs in vitro, and acts as a chemoattractant to outgrowing neurites (Hearn et al., 1998; Natarajan et al., 2002; Taraviras et al., 1999; Young et al., 2001). The mature ENS contains sensory neurons, interneurons and excitatory and inhibitory motor neurons that use a wide range of neurotransmitters, similar to those found in the CNS (Hao and Young, 2009).

Mutations in *RET* and *GDNF* in humans contribute to Hirschsprung disease (HSCR), a congenital disorder that is characterized by intestinal aganglionosis in a variable segment of the distal gut. HSCR patients present with tonic contraction of the muscle layers in the affected segment, which consequently leads to dilatation of the proximal bowel. The prevalence of HSCR is 1:5000 live births and it is considered an inherited disease. Based on the families reported, the mode of inheritance can differ. Most non-syndromic familial HSCR cases show a dominant pattern of inheritance, mostly with reduced penetrance, whereas many syndromic HSCR families show a recessive pattern of inheritance. The sporadic cases are considered polygenic or oligogenic. Of the approximately 15 HSCR genes identified so far, the *RET* gene is by far the most important gene. Coding mutations in *RET* are found in ~50% of familial and 15–35% of sporadic HSCR patients and represent the great majority of genetic mutations found in HSCR patients (Attié et al., 1995; Hofstra et al., 2000). Mutations in all other HSCR-associated genes, including *GDNF*, are rare (Alves et al., 2013; Amiel et al., 2008; Brooks et al., 2005a).

The colonization of the intestine by ENCCs, followed by their neuronal and glial differentiation, is a complex process controlled by various signaling pathways. These include Hedgehog, NOTCH, WNT, Retinoic acid and TGF-β/BMP signaling (Goldstein et al., 2005; Ikeya et al., 1997; Okamura and Saga, 2008; Ramalho-Santos et al., 2000; Sato and Heuckeroth, 2008). Several other pathways that are important for ENS development have been identified in gene expression profiling studies. Iwashita et al. (2003) reported that the HSCR genes *Ret*, *Gfrα1*, *Ednrb* and *Sox10* are highly expressed by ENCCs in mice, thereby explaining how mutations in these genes contribute to aganglionosis. A subsequent expression study by Heanue and Pachnis (2006) showed that at E15.5 in mice, ENCCs express markers of early and late neuronal development and glial differentiation, representing different stages of ENS development. These authors identified *Sox2*, *Cart*, *Sema*, *Fgf* and *Jnk* as novel signaling pathways in ENCCs (Heanue and Pachnis, 2006). In addition, Vohra et al. (2006) found that E14 mouse ENCCs highly express genes with synaptic functions and showed that these genes are important for ENCC migration and neurite outgrowth. Some insight in the pathways downstream of RET signaling has come from expression profiling of GDNF-treated ENCCs by Ngan et al. (2008), who analyzed the changes in gene expression in ENCCs after 8 and 16 h of GDNF treatment. These authors showed that the Prokineticin pathway is activated by RET signaling, but found that few other genes were differentially expressed by the short-term GDNF treatment of ENCCs (Ngan et al., 2008).

These gene expression studies have focused on individual genes that are highly expressed by ENCCs and showed that some of these genes are critical for the development of the ENS. However, for many highly expressed genes it is still unknown if and how they contribute to ENS development. In this study, we used an unbiased, systematic pathway analysis to identify the biological processes that are important for ENS development. Moreover, we treated ENCC cultures with GDNF for 14 days to identify the stable, long-term gene expression changes induced by RET signaling. We show that the expression of differentially expressed genes is regulated by known ENS-related genes, and by several genes not previously implicated in ENS development. These regulatory genes present novel candidates genes for HSCR.

2. Materials and methods

2.1. Animals

Male C57BL/6 mice carrying Wnt1-Cre$^{+/-}$ were mated with C57BL/6 females that were homozygous for the Rosa26-LoxP-Stop-LoxP-YFP reporter locus. The stage of embryonic development was set to E0.5 on the day the vaginal plug was seen. Embryos carrying both Wnt1-Cre and Rosa26-LoxP-Stop-LoxP-YFP were identified based on YFP expression in the gastrointestinal tract and craniofacial tissues.

2.2. ENCC isolation, culture conditions and RET activation

Full-length guts (foregut to hindgut) were dissected from E14.5 embryos. Single cell suspensions were made using 0.05 mg/ml Collagenase XI/Dispase II (Sigma Aldrich, St. Louis, MO, USA), followed by incubation for 3–5 min at 37 °C and trituration. YFP-positive ENCCs were FACS sorted and directly seeded in Fibronectin-coated plates containing DMEM/F-12 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 1% N2 supplement (Invitrogen), 2% B27 supplement (Invitrogen), 20 ng/ml FGF (Peprotech EC, London, UK), 20 ng/ml EGF (Peprotech EC) and 1% Penicillin-Streptomycin (Invitrogen). YFP-positive ENCCs from one gut were equally divided over two wells; one of which was supplemented with 50 ng/ml GDNF (Peprotech EC). ENCCs were incubated at 37 °C for 14 days in a humidified atmosphere with 5% CO_2. Neurosphere-like bodies appeared after 3–5 days and were kept in culture for 14 days without passaging.

2.3. Immunostaining

Wells with neurosphere-like bodies were fixed with 4% PFA for 15 min at room temperature (RT) and washed with PBS + 1%Triton X 100 (PBT) for 2 × 5 min. They were incubated in blocking solution (PBT + 1%BSA + 0.15% glycine) for 1 h at RT followed by primary antibodies diluted in blocking solution overnight (O/N) at 4 °C: rabbit anti-human p75 (1:500, Promega, UK), mouse-anti βIII-Tubulin (1:500, Covance UK). After several washes in PBT, secondary antibodies were applied: goat anti-rabbit Alexa Fluor 568 and goat anti-mouse Alexa Fluor 568 (1:500, Invitrogen) O/N. DAPI was added to secondary antibodies (1:1000). Antibody was washed off (3 × 15 min with PBT). Slides were mounted using Vectashield (Vector labs, UK) and visualized using confocal microscopy imaging on Zeiss LSM 710 (Zeiss, Cambridge, UK).

2.4. RNA isolation and gene expression quantification

RNA was isolated from untreated and GDNF-treated ENCC cultures, as well as from uncultured E14.5 embryonic guts using the RNeasy Mini Kit (Qiagen, Crawel, UK). RNA yield, purity and integrity were determined using the Agilent 2100 BioAnalyzer with 6000 Nano Chips (Agilent Technologies, Amsterdam, The Netherlands). DNA microarray analyses and RNA quality controls were performed by ServiceXS (Leiden, The Netherlands) using GeneChip Mouse Genome 430 2.0 arrays (Affymetrix, Santa Clara, CA, USA). For the ENCC vs gut experiment, 3 ENCC samples and 8 mouse gut samples entered the analysis. For the ENCC + GDNF vs ENCC experiment, 7 pairs of untreated and GNDF-treated ENCC cultures were analyzed.

2.5. Data normalization, statistical analysis and functional analysis

Quantile normalization and background correction were applied to the raw intensity values of all samples using Partek version 6.4 (Partek Incorporated, St. Louis, MO, USA) via GC Robust Multichip Analysis. For the ENCC vs gut analysis, 2 sample *T* test statistics were applied in Partek to calculate the fold changes with associated p-values. Paired *T* test statistics were applied to calculate the fold changes with associated p-values for the ENCC+GDNF vs ENCC analysis. The fold change and p-value data were uploaded into Ingenuity Pathway Analysis (IPA) (Ingenuity/Qiagen, Redwood City, CA, USA). The probe level data was mapped

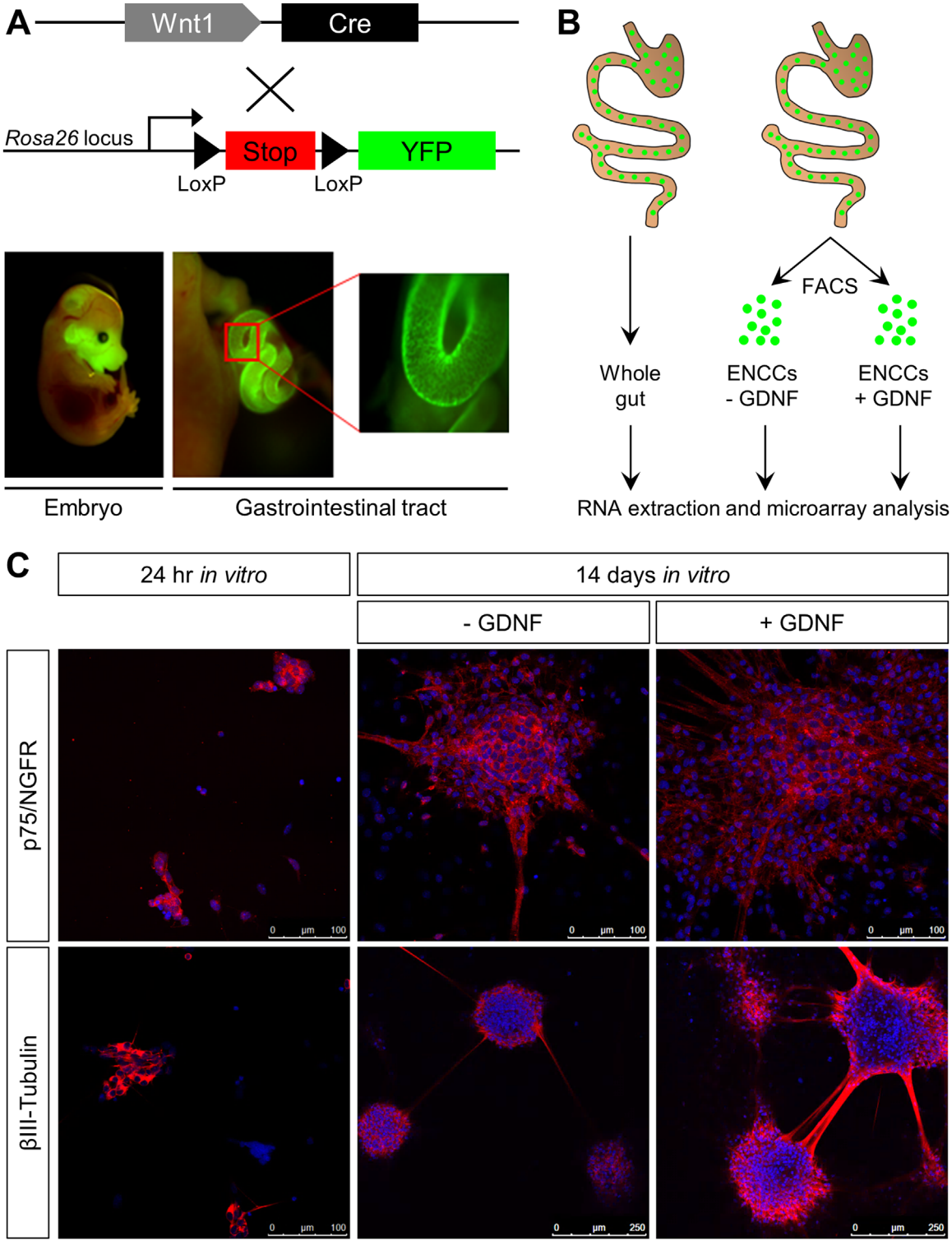


Fig. 1. Isolation and characterization of ENCCs. A) Schematic overview of the Wnt1-Cre:Rosa26-YFP mouse model. At embryonic day 14.5, transgenic mouse embryos expressed YFP in neural crest-derived cells, including craniofacial tissue and ENCCs. B) YFP-expressing ENCCs were FACS-sorted from dissected guts and were cultured in the presence or absence of GDNF for 14 days. RNA was isolated from ENCCs as well as whole gut tissue and relative RNA expression levels were quantified by microarray analysis. C) ENCCs expressed the neural crest marker p75/NGFR at both 24 h and 14 days after isolation. Pronounced βIII-Tubulin-positive connections between NLBs were observed in ENCC cultures treated with GNDF.

to the gene level and averaged based on the median fold change values. For the differential gene expression analysis, significance thresholds of fold change >5 and p-value $<1E-5$ were applied for the ENCC vs gut comparison. For the ENCC+GDNF vs ENCC analysis, significance thresholds were set at fold change >1.8 and p-value <0.05. Gene Ontology enrichment was performed using DAVID Bioinformatics Resources 6.7 and Upstream Regulator Analysis was performed in IPA. For the Upstream Regulator Analysis the significance thresholds were set to z-score >1.8 and p-value <0.05. Hierarchical clustering of Upstream Regulators and their downstream target genes was performed using the heatmap functionality in the HIV sequence database (http://www.hiv.lanl.gov).

2.6. Quantitative real-time PCR

Gastrointestinal tracts from E14.5 Wnt1-Cre:Rosa26-YFP mice were dissociated and FACS-sorted as describe above. YFP-positive cells were collected in 100 µl TRIzol reagent (Life Technologies, UK) and stored at −80 °C until RNA extraction. Guts from non-YFP embryos from the litter were also dissected out and frozen in TRIzol Reagent. Once thawed, samples were homogenized and RNA was extracted according to manufacturer's instructions. cDNA was synthesized using the iScript cDNA Synthesis kit (Bio-Rad). Quantitative real-time (qRT)-PCR primers were designed in Primer-BLAST software. KAPA SYBR FAST qPCR Kit (Kapa Biosystems) was loaded with primers and cDNA samples to a 96-well Hard-Shell PCR plate (Bio-Rad). qRT-PCR was carried out on a CFX96 Touch Real-Time PCR Detection System (Bio-Rad).

3. Results

3.1. Isolation of Enteric Neural Crest Cells

To identify genes that are involved in ENS development, gene expression profiles of pure populations of ENCCs were generated. Mice expressing Cre recombinase under the neural crest-specific Wnt1 promoter were crossed with mice carrying the Rosa26-LoxP-Stop-LoxP-YFP locus. ENCCs were isolated at embryonic day E14.5, since at this stage ENCCs express markers of both early and late ENS development (Heanue and Pachnis, 2006). Double transgenic mouse embryos expressed YFP in neural crest cells in craniofacial and gastrointestinal tissues as expected (Fig. 1a). YFP-expressing cells were isolated from E14.5 gut using Fluorescence activated cell sorting (FACS) (Fig. 1b). In culture, these cells formed neurosphere-like bodies (NLBs) within 3–5 days (Fig. 1c). Notably, NLBs from GDNF-treated ENCCs were larger than NLBs from untreated ENCCs. Compared to untreated cultures, GDNF-treated ENCC cultures showed marked βIII-Tubulin-positive connections between NLBs, indicating that GDNF treatment induced neuronal differentiation of ENCCs (Fig. 1c). Both untreated and GDNF-treated ENCC cultures expressed the neural crest marker p75/NGFR at both 24 h and 14 days in vitro.

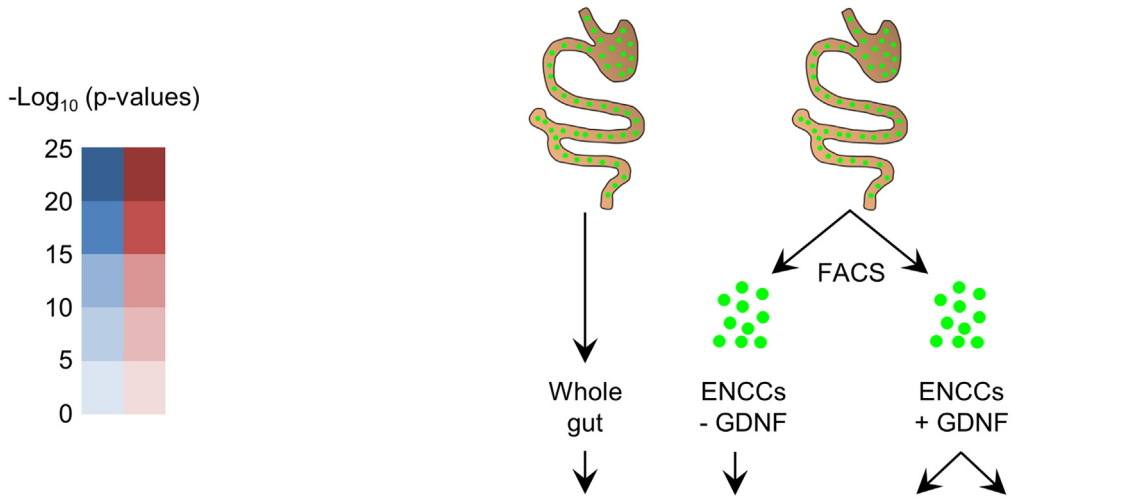
3.2. E14.5 ENCCs are enriched for markers of neuronal development and neuronal signaling

The gene expression profile of cultured ENCCs (harvested from E14.5 embryos) was compared to E14.5 embryonic gut to identify genes that are highly expressed by ENCCs. Since whole gut samples contain a variety of cell types, such as endothelial cells, smooth muscle, connective tissue, serosa, enterochromaffin cells and approximately 5% ENCCs, the expression profiles of ENCCs and whole gut tissue are expected to be very different. Therefore, only genes with an >5-fold change in gene expression (975 genes) were included in downstream analyses. Gene Ontology analysis was performed to identify the biological pathways these genes are involved in. ENCC-expressed genes were highly enriched for the Gene Ontology terms *transmission of nerve impulse*, *synaptic signaling* and *cell-cell signaling*, that are associated with neuronal signaling (Fig. 2). In addition, genes that are highly expressed in ENCCs play a role in *biological-/cell adhesion* and *neuron differentiation*. 1187 genes were >5 times less abundantly expressed in ENCCs than in the gut and these genes are involved in cell cycle control, in particular during active cell division (M-phase). Although the culturing period may have affected the expression of cell cycle genes in the ENCCs, this finding is consistent with the high proliferation rate of cells in the developing E14.5 mouse gut.

Interestingly, genes highly expressed by ENCCs (and low in gut tissue) and genes highly expressed in total gut tissue (and low in ENCCs), are both significantly enriched for Gene Ontology terms *biological-* and *cell adhesion* and *regulation of cell proliferation* (Fig. 2). However, these represent sets of genes with different properties and localizations. Adhesion molecules in ENCCs included genes expressed in the ENS, such as Immunoglobulin superfamily cell adhesion molecules (*Ncam1*, *Ncam2*, *L1cam*, *Nrcam*), (proto-)cadherins (*Cdh2*, *Cdh6*, *Pcdh15*, *Pcdha4*) and integrins (*Itga4*) (McKeown et al., 2013). Of note, *Cdh13* was highly expressed by ENCCs and its human orthologue *CDH13* maps to the HSCR susceptibility locus 16q23.3 (Carrasquillo et al., 2002). Adhesion molecules that were highly expressed in total gut tissue included *Cdh1*, which is expressed by Paneth cells and goblet cells in the intestinal epithelium (Schneider et al., 2010), the Claudin family of tight junction molecules (*CLDN 2, 3, 4, 5, 6, 7, 8, 15* and *23*), as well as genes involved in the apical junction complex (*Desmocollin 2*, *Desmoglein 2*, *Plakophilin 2* and *3*). These gut-expressed junction molecules are most likely expressed by the enterocytes of the intestinal epithelium.

3.3. GDNF treatment of ENCCs induces terminal neuronal differentiation

RET signaling is critical during all stages of ENS development and is important for survival, neuronal differentiation and neurite outgrowth at E14-E16 (Chalazonitis et al., 1998; Hearn et al., 1998; Natarajan et al., 2002; Taraviras et al., 1999; Young et al., 2001). To identify the molecular pathways underlying these processes, the changes in gene expression in ENCCs after 14 days of GDNF treatment were analyzed. Compared to untreated ENCCs, the expression of 445 genes was at least 1.8 times higher in GDNF-treated ENCCs. The neuronal signaling genes that were highly expressed by ENCCs compared to the gut were even further up-regulated upon GDNF treatment (Fig. 2). This finding suggests that GDNF treatment induced terminal neuronal differentiation in E14.5 ENCCs and is consistent with the pronounced βIII-Tubulin-positive connections that were observed between NLBs grown from GDNF-treated ENCCs (Fig. 1c). Additionally, the expression levels of 352 genes was at least 1.8 times reduced by treatment with GDNF, including genes involved in *biological-* and *cell adhesion* and *neuron differentiation*. The adhesion molecules that are down-regulated by GDNF include *Cdh13*, *Col5a1*, *Fibronectin-1*, *Reelin*, *Thrombospondin 1* and *4*, and *Vcam1*. These adhesion molecules are involved in *cell migration* and *cell motility*, and thus corroborate the observation that ENCCs stop migrating when they undergo terminal neuronal differentiation (Young et al., 2004, 2005). Consistent with its proliferative role in ENCCs and its inhibitory effect on neuronal differentiation, endothelin receptor type B (*Ednrb*) expression was down-regulated by GDNF. The down-regulation of genes involved in *neuron differentiation* and up-regulation of neuronal signaling genes, shows that different genes are involved in early and late neuronal development.



Gene Ontology term	Low in ENCCs (1187 genes)	High in ENCCs (975 genes)	Down by GDNF (352 genes)	Up by GDNF (445 genes)
Transmission of nerve impulse		6E-24		2E-10
Synaptic transmission		7E-23		1E-11
Cell-cell signaling		4E-22		2E-11
Biological adhesion	6E-05	4E-13	4E-09	
Cell adhesion	6E-05	5E-13	5E-09	
Neuron differentiation		7E-13	1E-05	
Ion transport		2E-07		6E-05
Vesicle-mediated transport		3E-05		7E-05
Regulation of cell proliferation	1E-04	1E-02	1E-08	
Skeletal system development		3E-02	1E-08	
Regulation of secretion		4E-02		3E-05
Cell cycle	6E-16			
M phase	8E-16			
Cell cycle phase	8E-16			
M phase of mitotic cell cycle	1E-15			
Nuclear division	9E-15			
Mitosis	9E-15			
Tube development	1E-08		1E-09	
Regulation of transcription from RNA pol II promoter	5E-03		1E-07	

Fig. 2. Gene Ontology analysis of differentially expressed genes. Differentially expressed genes between ENCCs and the gut, and between untreated and GDNF-treated ENCCs were annotated by Gene Ontology enrichment analysis. Values represent multiple testing corrected (Benjamini) p-values. The six most significant Gene Ontology terms per gene set are given and these are partially overlapping between the different sets of genes.

The mature ENS displays a great diversity in neuronal subtypes, but the molecular mechanisms controlling neuronal subtype specification in the ENS are poorly understood. To test whether RET signaling in ENCCs contributes to the specification of neuronal subtypes, the expression of neuronal subtypes markers was determined in untreated and GDNF-treated ENCCs. The expression levels of cholinergic (*Acat2*, *Ache*, *Chrna5*), glutamatergic (*Grik2*, *Gria1*, *Grid2*) and GABAergic (*Gabra1*, *Gabrg2*, *Gabrg3*) markers were increased by GDNF, as well as the expression of CART prepropeptide (*Cartpt*), NPY receptor Y2 (*Npy2r*), Substance P precursor *Tac1*, Dopamine beta-hydroxylase (*Dbh*) and Vasoactive intestinal peptide (*Vip*). The elevated expression of these subtype markers supports our observation that RET signaling is involved in terminal neuronal differentiation of ENCCs. Several other neuronal subtype markers, such as 5-HT, Calbindin, Calretinin, CGRP, Enkephalin and NOS were not differentially expressed. The wide range of neuronal subtypes markers that are up-regulated by GDNF suggests that RET signaling might be involved in the specification of different, but not all, neuronal subtypes.

3.4. Upstream Regulator Analysis identifies known and new genes important for ENS development

Having identified gene expression patterns in untreated and GDNF-treated ENCCs, we next aimed to identify regulatory genes in ENCCs and in GDNF-induced neuronal maturation. To address this, we used the Upstream Regulator Analysis tool in Ingenuity Pathway Analysis (IPA). Upstream Regulators are predicted to

Table 1
Upstream Regulators of gene expression in naïve and GDNF-treated ENCCs. Regulatory genes in ENCCs were predicted by IPA Upstream Regulator Analysis. Significant Upstream Regulators were defined as having an activation z-score > 1.8 and p-value of overlap < 0.05. From the Upstream Regulator Analysis, genes were selected that were expressed > 5 times higher in ENCCs than in the gut, or that were > 1.8 times up-regulated by GDNF. The Upstream Regulators can be linked to ENS development and HSCR (also see Fig. 5).

Dataset	Upstream Regulator	Activation z-score	p-Value of overlap	Target genes	Fold change	Link to HSCR
High in ENCCs	*Cdkn2a*	5.196	1.65E−07	55	467.6	
	Nupr1	4.740	2.51E−16	127	6.2	
	Bdnf	3.286	1.37E−05	46	9.2	Regulator of SNAP25 and VAMP2; migration of ENCCs[a]
	App	2.931	1.47E−12	157	7.1	Overexpression (Down syndrome) causes neuronal loss[b] Regulator of SNAP25 and VAMP2; migration of ENCCs[a] Inhibitor of MMP2 that is important for migration of ENCCs[c]
	Sox10	2.596	1.56E−03	9	19.8	Known HCSR gene[d]
	Ret	2.359	9.10E−05	29	16.5	Known HSCR gene[e]
	Mapk10	2.195	6.71E−03	5	22.7	Deletion in HSCR patient[f] Phosphorylates APP and STMN2[g]
	Elavl1	2.183	1.98E−02	16	5.4	
	Timp1	1.890	1.38E−02	7	7.9	
Up by GDNF	*Adora2a*	1.941	8.39E−02	10	1.9	
	Npy2r	1.727	2.58E−03	4	1.9	Maps to HSCR susceptibility locus 4q31.3-q32.3[h]

[a] Vohra et al. (2006).
[b] Salehi et al. (2006), Trazzi et al. (2013) and Semar et al. (2013).
[c] Anderson (2010).
[d] Pingault et al. (1998).
[e] Edery et al. (1994) and Romeo et al. (1994).
[f] Jiang et al. (2011).
[g] Kimberly et al. (2005) and Neidhart et al. (2001).
[h] Brooks et al. (2006).

regulate the expression of differentially expressed genes, taking into account the enrichment for genes that are known to be regulated by a particular Upstream Regulator (*p*-value of overlap) and the direction of differential gene expression (activation z-score) (Krämer et al., 2014). Many genes that are known to have important regulatory functions in ENS development are highly expressed by ENCCs. Therefore, genes were selected from the Upstream Regulator Analysis that were either highly expressed by ENCCs or up-regulated by GDNF. The Upstream Regulator Analysis of differentially gene expression between ENCCs and the gut yielded nine regulatory genes that are highly expressed by ENCCs themselves (Table 1).

These Upstream Regulators were expressed in ENCCs that were cultured in vitro for 14 days. To confirm that these genes are endogenously expressed in ENCCs, qRT-PCR analysis was performed on RNA from acutely isolated, uncultured ENCCs from E14.5 embryonic gut. The cell cycle-associated genes *Elavl1* and *Nupr1* were not differentially expressed between uncultured ENCCs and whole

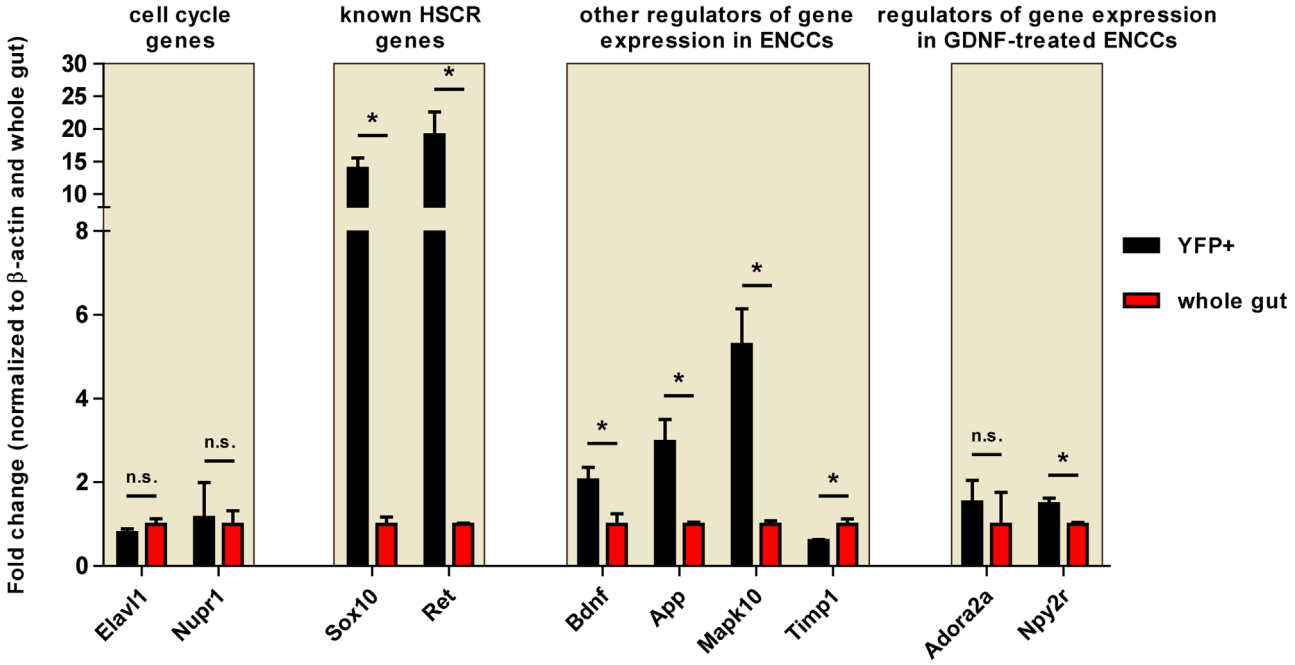


Fig. 3. Expression of the Upstream Regulators in acutely isolated ENCCs. Except for *Cdkn2a*, all predicted Upstream Regulators were detected by qRT-PCR analysis on acutely isolated, uncultured ENCCs from E14.5 embryonic gut. The expression levels of *Elavl1*, *Nupr1* and *Timp1* were not higher in uncultured ENCCs than in whole gut, suggesting that the high expression of these genes in the microarray data was induced by culturing. The high expression of *Sox10*, *Ret*, *Bdnf*, *App* and *Mapk10* was confirmed in acutely isolated, uncultured cells. *Adora2a* and *Npy2r* were near-significant regulators of gene expression in GDNF-treated ENCCs and both genes were expressed in acutely isolated ENCCs. *Benjamini-Hochberg adjusted p-value < 0.05.

gut samples (Fig. 3). *Timp1*, although expressed in acutely isolated ENCCs, was expressed in ENCCs at lower levels than in whole gut. *Cdkn2a* expression levels were below the detection limit, suggesting that the gene was very lowly or not expressed in uncultured ENCCs. These data suggest that the high gene expression levels of *Elavl1*, *Nupr1*, *Timp1* and *Cdkn2a* in cultured ENCCs was due to the culture conditions.

The Upstream Regulators with a high expression in acutely isolated ENCCs compared to whole gut were *Sox10*, *Ret*, *Bdnf*, *App* and *Mapk10*. Of these genes, *Sox10* and *Ret* are well-established ENS development genes. SOX10 is a transcription factor that is required for survival of ENCCs and maintenance of their progenitor state (Bondurand et al., 2006; Kapur, 1999). The identification of *Ret* and *Sox10* as regulatory genes in ENCCs demonstrates the validity of the Upstream Regulator Analysis and suggests that the remaining Upstream Regulators and their respective pathways are key regulators of ENS development. These remaining Upstream Regulators are *Bdnf*, *App* and *Mapk10*.

Brain Derived Neurotrophic Factor (*Bdnf*) is expressed by ENCCs and the mucosa and its receptors TRKB and p75/NGFR are abundantly expressed in the human and mouse ENS (Hoehner et al., 1996; Lommatzsch et al., 1999). BDNF signaling promotes neuronal survival, growth and differentiation and functions in the mature ENS by controlling peristalsis and colonic emptying (Coulie et al., 2000; Grider et al., 2006).

Amyloid Precursor Protein (*App*) is the precursor of Amyloid-β plaques in Alzheimer disease. The endogenous function of APP is not completely understood, but APP has been shown to be involved in synapse formation and plasticity and is expressed in the human ENS (Arai et al., 1991; Cabal et al., 1995; Turner et al., 2003; Tyan et al., 2012).

Mitogen-Activated Protein Kinase 10 (*Mapk10/Jnk3*) is highly expressed by ENCCs (Heanue and Pachnis, 2006). MAPK10 is involved in a broad range of processes, including proliferation, differentiation, inflammation, cellular stress and neuronal apoptosis and is regulated by RET signaling in ENCCs (Ngan et al., 2008).

3.5. Adenosine and neuropeptide Y signaling modulate GDNF-induced changes in gene expression

Upstream Regulator Analysis of GDNF-induced changes in gene expression did not result in any genes that have both a significant activation z-score and p-value. There are, however, two Upstream Regulators with near-significant z-scores and p-values; the adenosine receptor A2a (*Adora2a*) and the neuropeptide Y receptor Y2 (*Npy2r*). *Adora2a* and *Npy2r* were both expressed in acutely isolated ENCCs from E14.5 embryos and *Npy2r* was more abundantly expressed in ENCCs than in whole gut (Fig. 3). Adenosine regulates intestinal secretion, motility and immune responses and acts through adenosine receptors A1, A2a, A2b and A3. The adenosine A2a receptor is expressed by the myenteric- and submucosal plexuses in both the small and large intestine (Antonioli et al., 2008). ADORA2A modulates intestinal motility by inhibiting motor activity in the duodenum and colon of mice and humans, respectively (Fornai et al., 2009; Zizzo et al., 2011), thereby allowing relaxation of the bowel.

Npy2r encodes the neuropeptide Y receptor Y2, a receptor for neuropeptide Y (NPY) and peptide YY (PYY) (Chen et al., 1997). NPY and PYY induce colonic smooth muscle relaxation and inhibit intestinal contractility and motility in response to feeding, which is exerted through the neuropeptide Y1 and Y2 receptors (Hellström, 1987; Tough et al., 2011).

3.6. Synergistic regulation of gene expression

The predicted Upstream Regulators each have between 4 and 157 downstream targets, combining up to 213 unique genes. Although a single Upstream Regulator regulates the expression of most of these genes, there are also 38 genes that are regulated by multiple regulatory genes (Fig. 4). Many of the genes that are regulated by three or more Upstream Regulators are known to play a role in the ENS, such as *App*, *c-Jun*, *Nos1*, *IL-6*, *Napb*, *(p75/)Ngfr* and *Npy*. Hierarchical clustering of downstream target genes showed that there are clusters of co-regulated genes with similar functions. For example, *Bdnf*, *Gap43*, *Mapt*, *Mbp*, *Penk*, *Snap25*, *Syn1*, *Syn2*, *Syp*, *Tubb3* and *Vamp2* all are involved in synaptic signaling and are synergistically regulated by *Bdnf* and *App*. Likewise, *Prnp*, *Klk3*, *D cn* , *Calb1*, *Ccl2*, *Cd151*, *Csf1*, *Ctsb*, *Cxcl12*, *Dpysl2* and *Stmn2* are regulated by both *Ret* and *App*. The synergistic regulation of downstream target genes by multiple Upstream Regulators provides insight into the molecular networks underlying ENS development.

4. Discussion

In this study we have analyzed gene expression profiles of GDNF-treated and untreated mouse ENCCs (isolation time E14.5) and E14.5 mouse embryonic gut. Unbiased, systematic Gene Ontology analysis showed that, compared to the whole gut, ENCCs highly express genes involved in cell adhesion, neuronal differentiation and synaptic signaling, representing various aspects of ENS development. These data corroborate findings by Heanue and Pachnis (Heanue and Pachnis, 2006). These authors compared gene expression profiles of E15.5 ganglionic ($Ret^{+/+}$) and aganglionic ($Ret^{k-/k-}$) gut and found that ENCCs express markers of early differentiating glia and neurons, terminally differentiated neurons, axonal outgrowth and synaptogenesis. In fact, of the 47 genes that were confirmed to be expressed in the ENS by in situ hybridization in that study, 35 genes (74%) were highly expressed by ENCCs in our study. Likewise, the gene expression study by Vohra et al. confirmed expression in the ENS for 38 genes by in situ hybridization, of which 23 genes (61%) were differently expressed between ENCCs and the gut in the present study (Vohra et al., 2006). Of the 105 ENCC-expressed genes found by Iwashita et al., 19 genes (18%) overlap with our data (Iwashita et al., 2003). Importantly, this shows that the 14 days in vitro culturing of ENCCs in our study did not lead to major changes in gene expression. Of the 975 genes that were expressed higher in ENCCs than in total gut tissue, 848 genes (87%) were not reported in any of the three expression studies mentioned above. However, part of these 848 genes are already reported to be involved in ENS development (Kuo et al., 2010; Teng et al., 2008). This study therefore represents a more complete catalog of known and newly identified genes that are expressed in the developing ENS.

4.1. Regulators of gene expression in ENCCs are putative HSCR genes

Sox10, *Ret*, *Bdnf*, *App* and *Mapk10* were identified as genes that are highly expressed in ENCCs and that regulate the expression of other ENCC-expressed genes. Of these regulators, *RET* and *SOX10* are known HSCR genes. Mutations in *RET* and *SOX10* contribute to HSCR in humans and mice with null mutations for *Ret* and *Sox10* display total intestinal aganglionosis (Edery et al., 1994; Herbarth et al., 1998; Pingault et al., 1998; Romeo et al., 1994; Schuchardt et al., 1994; Southard-Smith et al., 1998). Given the known neuronal functions of *Bdnf*, *App*, and *Mapk10*, this raises the hypothesis that these regulatory genes might also be implicated in HSCR and other neuropathies, such as intestinal pseudo-obstruction, incontinence or constipation.

Mapk10 is a highly interesting regulator of gene expression in ENCCs with regard to HSCR. MAPK10 modulates neuronal

Fig. 4. Upstream Regulators regulate gene expression synergistically. The regulation of downstream target genes (right-hand side) by Upstream Regulators (bottom) is shown for all genes that are regulated by two or more Upstream Regulators. Red and blue boxes indicate regulation by an Upstream Regulator in naïve and GDNF-treated ENCCs, respectively. Yellow indicates that the gene is not regulated by the specific Upstream Regulator.

differentiation through phosphorylation of APP and STMN2 (Kimberly et al., 2005; Neidhart et al., 2001). STMN2 (SCG10) interacts with Kinesin Binding Protein (KBP, KIAA1279) and mutations in *KBP* have been linked to the HSCR-associated Goldberg-Shprintzen Syndrome (Alves et al., 2010; Brooks et al., 2005b). Loss of MAPK10 could therefore contribute to HSCR through impaired APP and/or STMN2 signaling in enteric neuron differentiation. Since *Mapk10* was reported to be highly expressed in ENCCs, it was included in a copy number variant screen for candidate genes in HSCR (Jiang et al., 2011). The authors reported one deletion in *MAPK10* among 18 HSCR patients. Segregation analysis showed that the *MAPK10* deletion was neither necessary nor sufficient to cause HSCR in this family and the authors therefore hypothesized that the deletion in *MAPK10* acts as a modifier for developing HSCR (Jiang et al., 2011).

ENS development is of interest in relation to Down syndrome (Trisomy 21). The incidence of HSCR in Down syndrome is 2.6%, which is over a hundred times higher than in the general population (Friedmacher and Puri, 2013). This suggests that the duplication of one or more genes on chromosome 21 contributes to the onset of HSCR. *APP* is located on chromosome 21 in humans and *App* is an Upstream Regulator of gene expression in the developing murine ENS. It has been shown that overexpression of wild type human *APP* in mice causes loss of cholinergic neurons through disruption of Nerve Growth Factor transport and impairs neurogenesis in the CNS (Salehi et al., 2006; Trazzi et al., 2013). Moreover, mice overexpressing an Alzheimer's disease mutant form of *APP* show reduced neuronal density in the myenteric plexus and altered intestinal motility (Semar et al., 2013). In the latter study it is not clear, however, whether the phenotype was caused by the increased expression level of *APP* or by the presence of the Alzheimer mutation in the overexpressed gene. APP contains an inhibitor domain that inhibits matrix metalloproteinase 2 (MMP2) (Miyazaki et al., 1993) and pharmacological inhibition of MMP2 impairs the migration of ENCCs along the mouse gut (Anderson, 2010). Inhibition of MMP2 is another mechanism by which overexpression of APP might contribute to the development of HSCR in Down syndrome. These reports, combined with the regulatory function of *App* in ENCCs, raise the intriguing hypothesis that overexpression of *APP* might contribute to the high incidence of HSCR and other gastrointestinal abnormalities in Down syndrome patients.

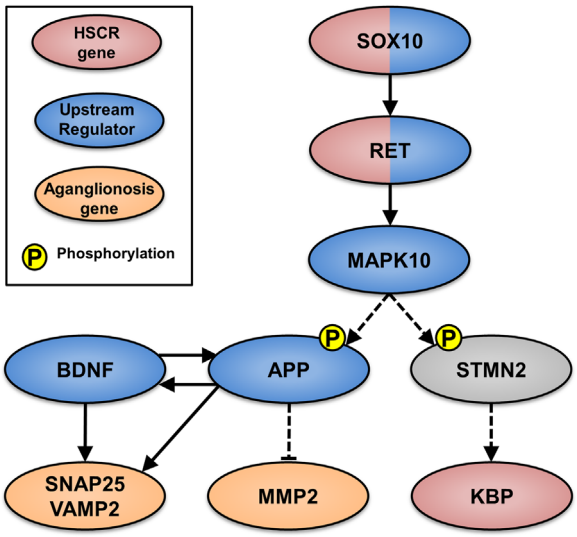


Fig. 5. Hypothetical molecular network implicating Upstream Regulators in HSCR. The Upstream Regulators in ENCCs are either known HSCR genes (*RET* and *SOX10*), can be linked to a known HSCR gene (*KBP*), or can be linked to genes that are critical for colonization of the gut by ENCCs (*SNAP25*, *VAMP2* and *MMP2*). The link of each Upstream Regulator to HSCR and the respective references are summarized in Table 1. Arrows indicate activation and blunted lines indicate inhibition. Solid lines represent direct interactions in ENCCs. Dotted lines represent indirect interactions and evidence from other cell types.

In our Upstream Regulator Analysis, *App* and *Bdnf* shared several downstream target molecules that are involved in synaptic signaling, including *Snap25* and *Vamp2*. *Snap25* and *Vamp2* mediate the fusion of synaptic vesicles and the high expression of these genes in ENCCs was reported by Vohra et al., who subsequently showed that *Snap25* and *Vamp2* are important for ENCC migration and neurite outgrowth (Vohra et al., 2006). Since both genes are regulated by APP and BDNF, aberrant APP and BDNF signaling might result in similar defects in ENS development.

As discussed here, the Upstream Regulators with neuronal functions are either known HSCR genes, or are linked to the molecular pathways underlying HSCR or animal models of aganglionosis. There is a large body of literature suggesting interactions between these genes, allowing us to construct a hypothetical network of regulatory genes in ENCCs (Fig. 5). The transcription factor SOX10 binds to an enhancer of *Ret*, thereby regulating *Ret* expression (Lang and Epstein, 2003). In turn, RET activates the MAPK pathway, including MAPK10 (Ngan et al., 2008; Plaza-Menacho et al., 2006). MAPK10 phosphorylates APP, which regulates the expression of synaptic genes *Snap25* and *Vamp2* together with BDNF, and inhibits MMP2 activity (Kimberly et al., 2005; Miyazaki et al., 1993). MAPK10 also phosphorylates STMN2, leading to cytoskeletal remodeling in conjunction with KBP (Alves et al., 2010; Neidhart et al., 2001).

4.2. Modulators of RET-signaling in ENCCs

Several signaling cascades are activated by RET, including Pi3K/AKT, ERK, p38-MAPK and JNK pathways (Plaza-Menacho et al., 2006). Little is known, however, how these pathways contribute to RET-induced proliferation, migration, differentiation and survival of ENCCs. Similar to our study, Ngan et al. analyzed the changes in gene expression induced by GDNF treatment of ENCCs and found that the expression levels of 29 genes were increased by GDNF treatment. Only 4 of these 29 genes were up-regulated by GDNF in our data. These different findings can be explained by two fundamental differences in study design, duration of GDNF stimulation and developmental stage of ENCCs. Ngan et al. investigated the short-term changes (8 and 16 h GDNF treatment) in E11.5 ENCCs, when RET is required for migration and proliferation. In the present study, on the other hand, we have analyzed the long-term changes (14 days GDNF treatment) in ENCCs at E14.5, a stage in which RET is involved in survival and differentiation. The four genes that are up-regulated by GDNF treatment in both studies (*Kcnd2*, *Vip*, *Olfm1* and *Tagln3*), are therefore likely to be critical modulators of RET signaling and are possibly involved in different aspects of ENS development.

Long-term GDNF treatment of ENCCs resulted in reduced expression levels of genes involved in neuronal migration and early neuronal differentiation. Conversely, GDNF increased the expression of genes that are associated with terminal neuronal differentiation and synaptic signaling. Upstream Regulator Analysis showed that the adenosine receptor A2a and the neuropeptide Y receptor Y2 are near-significant modifiers of these cellular processes in GDNF-treated ENCCs. Signaling via NPY2R modulates the neurotrophic effect of GDNF on ENCCs, as a study by Anitha et al. (2006) has shown that GDNF treatment of ENCCs results in increased proliferation and reduced apoptosis of ENCCs in a NPY-dependent manner. This report is in line with our observation that NLBs from GDNF-treated ENCCs are larger than NLBs from untreated ENCCs. Of interest, the human orthologue of *Npy2r* maps to the HSCR susceptibility locus 4q31.3-q32.3 and increased NPY levels have been reported in aganglionic bowel segments from HSCR patients (Brooks et al., 2006; Hamada et al., 1987).

5. Concluding remarks

In conclusion, systematic analysis of E14.5 ENCCs gene expression profiles showed that they express markers of neural migration, axonal outgrowth and neuronal signaling. Upstream Regulator Analysis predicted genes that serve critical regulatory functions during ENS development and we propose a mechanistic network of action for these regulatory genes. Additionally, we found that GDNF treatment of ENCCs caused terminal neuronal differentiation into a variety of neuronal subtypes. These regulatory genes in ENS development give insight into the molecular pathways regulating ENS development and provide novel candidate genes for HSCR.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ydbio.2016.06.004.

References

Alves, M.M., Burzynski, G., Delalande, J.-M., Osinga, J., van der Goot, A., Dolga, A.M., de Graaff, E., Brooks, A.S., Metzger, M., Eisel, U.L.M., Shepherd, I., Eggen, B.J.L., Hofstra, R.M.W., 2010. KBP interacts with SCG10, linking Goldberg-Shprintzen syndrome to microtubule dynamics and neuronal differentiation. Hum. Mol. Genet. 19, 3642–3651. http://dx.doi.org/10.1093/hmg/ddq280.

Alves, M.M., Sribudiani, Y., Brouwer, R.W.W., Amiel, J., Antiñolo, G., Borrego, S.,

Ceccherini, I., Chakravarti, A., Fernández, R.M., Garcia-Barcelo, M.-M., Griseri, P., Lyonnet, S., Tam, P.K., van Ijcken, W.F.J., Eggen, B.J.L., te Meerman, G.J., Hofstra, R.M.W., 2013. Contribution of rare and common variants determine complex diseases-Hirschsprung disease as a model. Dev. Biol. 382, 320–329. http://dx.doi.org/10.1016/j.ydbio.2013.05.019.

Amiel, J., Sproat-Emison, E., Garcia-Barcelo, M., Lantieri, F., Burzynski, G., Borrego, S., Pelet, A., Arnold, S., Miao, X., Griseri, P., Brooks, A.S., Antinolo, G., de Pontual, L., Clement-Ziza, M., Munnich, A., Kashuk, C., West, K., Wong, K.K.-Y., Lyonnet, S., Chakravarti, A., Tam, P.K.-H., Ceccherini, I., Hofstra, R.M.W., Fernandez, R., 2008. Hirschsprung disease, associated syndromes and genetics: a review. J. Med. Genet. 45, 1–14. http://dx.doi.org/10.1136/jmg.2007.053959.

Anderson, R.B., 2010. Matrix metalloproteinase-2 is involved in the migration and network formation of enteric neural crest-derived cells. Int. J. Dev. Biol. 54, 63–69. http://dx.doi.org/10.1387/ijdb.082667ra.

Anitha, M., Chandrasekharan, B., Salgado, J.R., Grouzmann, E., Mwangi, S., Sitaraman, S.V., Srinivasan, S., 2006. Glial-derived neurotrophic factor modulates enteric neuronal survival and proliferation through neuropeptide Y. Gastroenterology 131, 1164–1178. http://dx.doi.org/10.1053/j.gastro.2006.07.019.

Antonioli, L., Fornai, M., Colucci, R., Ghisu, N., Tuccori, M., Del Tacca, M., Blandizzi, C., 2008. Regulation of enteric functions by adenosine: pathophysiological and pharmacological implications. Pharmacol. Ther. . http://dx.doi.org/10.1016/j.pharmthera.2008.08.010

Arai, H., Lee, V.M., Messinger, M.L., Greenberg, B.D., Lowery, D.E., Trojanowski, J.Q., 1991. Expression patterns of beta-amyloid precursor protein (beta-APP) in neural and nonneural human tissues from Alzheimer's disease and control subjects. Ann. Neurol. 30, 686–693. http://dx.doi.org/10.1002/ana.410300509.

Attié, T., Pelet, A., Edery, P., Eng, C., Mulligan, L.M., Amiel, J., Boutrand, L., Beldjord, C., Nihoul-Fékété, C., Munnich, A., 1995. Diversity of RET proto-oncogene mutations in familial and sporadic Hirschsprung disease. Hum. Mol. Genet. 4, 1381–1386.

Bondurand, N., Natarajan, D., Barlow, A., Thapar, N., Pachnis, V., 2006. Maintenance of mammalian enteric nervous system progenitors by SOX10 and endothelin 3 signalling. Development 133, 2075–2086. http://dx.doi.org/10.1242/dev.02375.

Brooks, A.S., Bertoli-Avella, A.M., Burzynski, G.M., Breedveld, G.J., Osinga, J., Boven, L.G., Hurst, J.A., Mancini, G.M.S., Lequin, M.H., de Coo, R.F., Matera, I., de Graaff, E., Meijers, C., Willems, P.J., Tibboel, D., Oostra, B.A., Hofstra, R.M.W., 2005a. Homozygous nonsense mutations in KIAA1279 are associated with malformations of the central and enteric nervous systems. Am. J. Hum. Genet. 77, 120–126. http://dx.doi.org/10.1086/431244.

Brooks, A.S., Oostra, B.A., Hofstra, R.M.W., 2005b. Studying the genetics of Hirschsprung's disease: Unraveling an oligogenic disorder. Clin. Genet. 67, 6–14. http://dx.doi.org/10.1111/j.1399-0004.2004.00319.x.

Brooks, A.S., Leegwater, P.A., Burzynski, G.M., Willems, P.J., de Graaf, B., van Langen, I., Heutink, P., Oostra, B.A., Hofstra, R.M.W., Bertoli-Avella, A.M., 2006. A novel susceptibility locus for Hirschsprung's disease maps to 4q31.3-q32.3. J. Med. Genet. 43, e35. http://dx.doi.org/10.1136/jmg.2005.038125.

Burns, A.J., Douarin, N.M., 1998. The sacral neural crest contributes neurons and glia to the post-umbilical gut: spatiotemporal analysis of the development of the enteric nervous system. Development 125, 4335–4347.

Cabal, A., Alonso-Cortina, V., Gonzalez-Vazquez, L.O., Naves, F.J., Del Valle, M.E., Vega, J.A., 1995. beta-Amyloid precursor protein (beta APP) in human gut with special reference to the enteric nervous system. Brain Res. Bull. 38, 417–423.

Carrasquillo, M.M., McCallion, A.S., Puffenberger, E.G., Kashuk, C.S., Nouri, N., Chakravarti, A., 2002. Genome-wide association study and mouse model identify interaction between RET and EDNRB pathways in Hirschsprung disease. Nat. Genet. 32, 237–244. http://dx.doi.org/10.1038/ng998.

Chalazonitis, a, Rothman, T.P., Chen, J., Gershon, M.D., 1998. Age-dependent differences in the effects of GDNF and NT-3 on the development of neurons and glia from neural crest-derived precursors immunoselected from the fetal rat gut: expression of GFRalpha-1 in vitro and in vivo. Dev. Biol. 204, 385–406. http://dx.doi.org/10.1006/dbio.1998.9090.

Chen, C.H., Stephens, R.L., Rogers, R.C., 1997. PYY and NPY: control of gastric motility via action on Y1 and Y2 receptors in the DVC. Neurogastroenterol. Motil. 9, 109–116.

Coulie, B., Szarka, L.A., Camilleri, M., Burton, D.D., McKinzie, S., Stambler, N., Cedarbaum, J.M., 2000. Recombinant human neurotrophic factors accelerate colonic transit and relieve constipation in humans. Gastroenterology, doi: S0016508500656135 (pii).

Druckenbrod, N.R., Epstein, M.L., 2005. The pattern of neural crest advance in the cecum and colon. Dev. Biol. 287, 125–133. http://dx.doi.org/10.1016/j.ydbio.2005.08.040.

Durbec, P., Marcos-Gutierrez, C.V., Kilkenny, C., Grigoriou, M., Wartiowaara, K., Suvanto, P., Smith, D., Ponder, B., Costantini, F., Saarma, M., 1996. GDNF signalling through the Ret receptor tyrosine kinase. Nature 381, 789–793. http://dx.doi.org/10.1038/381789a0.

Durbec, P.L., Larsson-Blomberg, L.B., Schuchardt, A., Costantini, F., Pachnis, V., 1996. Common origin and developmental dependence on c-ret of subsets of enteric and sympathetic neuroblasts. Development 122, 349–358.

Edery, P., Lyonnet, S., Mulligan, L.M., Pelet, A., Dow, E., Abel, L., Holder, S., Nihoul-Fékété, C., Ponder, B.A., Munnich, A., 1994. Mutations of the RET proto-oncogene in Hirschsprung's disease. Nature 367, 378–380.

Enomoto, H., Araki, T., Jackman, A., Heuckeroth, R.O., Snider, W.D., Johnson, E.M., Milbrandt, J., 1998. GFR alpha1-deficient mice have deficits in the enteric nervous system and kidneys. Neuron 21, 317–324. http://dx.doi.org/10.1016/S0896-6273(00)80541-3.

Fornai, M., Antonioli, L., Colucci, R., Ghisu, N., Buccianti, P., Marioni, a, Chiarugi, M., Tuccori, M., Blandizzi, C., Del Tacca, M., 2009. A1 and A2a receptors mediate inhibitory effects of adenosine on the motor activity of human colon. Neurogastroenterol. Motil. 21, 451–466. http://dx.doi.org/10.1111/j.1365-2982.2008.01213.x.

Friedmacher, F., Puri, P., 2013. Hirschsprung's disease associated with Down syndrome: a meta-analysis of incidence, functional outcomes and mortality. Pediatr. Surg. Int. 29, 937–946. http://dx.doi.org/10.1007/s00383-013-3361-1.

Furness, J.B., 2006. The Enteric Nervous System. Blackwell Publishing, Oxford.

Gershon, M.D., 2005. Nerves, reflexes, and the enteric nervous system: pathogenesis of the irritable bowel syndrome. J. Clin. Gastroenterol. 39, S184–S193.

Gianino, S., Grider, J.R., Cresswell, J., Enomoto, H., Heuckeroth, R.O., 2003. GDNF availability determines enteric neuron number by controlling precursor proliferation. Development 130, 2187–2198. http://dx.doi.org/10.1242/dev.00433.

Goldstein, A.M., Brewer, K.C., Doyle, A.M., Nagy, N., Roberts, D.J., 2005. BMP signaling is necessary for neural crest cell migration and ganglion formation in the enteric nervous system. Mech. Dev. 122, 821–833. http://dx.doi.org/10.1016/j.mod.2005.03.003.

Grider, J.R., Piland, B.E., Gulick, M.A., Qiao, L.Y., 2006. Brain-derived neurotrophic factor augments peristalsis by augmenting 5-HT and calcitonin gene-related peptide release. Gastroenterology 130, 771–780. http://dx.doi.org/10.1053/j.gastro.2005.12.026.

Hamada, Y., Bishop, A.E., Federici, G., Rivosecchi, M., Talbot, I.C., Polak, J.M., 1987. Increased neuropeptide Y-immunoreactive innervation of aganglionic bowel in Hirschsprung's disease. Virchows Arch. A Pathol. Anat. Histopathol. 411, 369–377.

Hao, M.M., Young, H.M., 2009. Development of enteric neuron diversity. J. Cell. Mol. Med. 13, 1193–1210. http://dx.doi.org/10.1111/j.1582-4934.2009.00813.x.

Heanue, T.A., Pachnis, V., 2006. Expression profiling the developing mammalian enteric nervous system identifies marker and candidate Hirschsprung disease genes. Proc. Natl. Acad. Sci. USA 103, 6919–6924. http://dx.doi.org/10.1073/pnas.0602152103.

Hearn, C.J., Murphy, M., Newgreen, D., 1998. GDNF and ET-3 differentially modulate the numbers of avian enteric neural crest cells and enteric neurons in vitro. Dev. Biol. 197, 93–105. http://dx.doi.org/10.1006/dbio.1998.8876.

Hellström, P.M., 1987. Mechanisms involved in colonic vasoconstriction and inhibition of motility induced by neuropeptide Y. Acta Physiol. Scand. 129, 549–556.

Herbarth, B., Pingault, V., Bondurand, N., Kuhlbrodt, K., Hermans-Borgmeyer, I., Puliti, A., Lemort, N., Goossens, M., Wegner, M., 1998. Mutation of the Sry-related Sox10 gene in Dominant megacolon, a mouse model for human Hirschsprung disease. Proc. Natl. Acad. Sci. USA 95, 5161–5165. http://dx.doi.org/10.1073/pnas.95.9.5161.

Hoehner, J.C., Wester, T., Pahlman, S., Olsen, L., 1996. Localization of neurotrophins and their high-affinity receptors during human enteric nervous system development. Gastroenterology 110, 756–767. http://dx.doi.org/10.1053/gast.1996.v110.pm8608885.

Hofstra, R.M., Wu, Y., Stulp, R.P., Elfferich, P., Osinga, J., Maas, S.M., Siderius, L., Brooks, A.S., vd Ende, J.J., Heydendael, V.M., Severijnen, R.S., Bax, K.M., Meijers, C., Buys, C.H., 2000. RET and GDNF gene scanning in Hirschsprung patients using two dual denaturing gel systems. Hum. Mutat. 15, 418–429. http://dx.doi.org/10.1002/(SICI)1098-1004(200005)15:5 < 418::AID-HUMU3 > 3.0.CO;2-2.

Ikeya, M., Lee, S.M., Johnson, J.E., McMahon, A.P., Takada, S., 1997. Wnt signalling required for expansion of neural crest and CNS progenitors. Nature 389, 966–970. http://dx.doi.org/10.1038/40146.

Iwashita, T., Kruger, G.M., Pardal, R., Kiel, M.J., Morrison, S.J., 2003. Hirschsprung disease is linked to defects in neural crest stem cell function. Science 301, 972–976. http://dx.doi.org/10.1126/science.1085649.

Jiang, Q., Ho, Y.-Y., Hao, L., Nichols Berrios, C., Chakravarti, A., 2011. Copy number variants in candidate genes are genetic modifiers of Hirschsprung disease. PLoS One 6, e21219. http://dx.doi.org/10.1371/journal.pone.0021219.

Kapur, R.P., 1999. Early death of neural crest cells is responsible for total enteric aganglionosis in Sox10(Do)m)/Sox10(Dom) (mouse embryos). Pediatr. Dev. Pathol. 2, 559–569. http://dx.doi.org/10.1007/s100249900162.

Kimberly, W.T., Zheng, J.B., Town, T., Flavell, R.A., Selkoe, D.J., 2005. Physiological regulation of the beta-amyloid precursor protein signaling domain by c-Jun N-terminal kinase JNK3 during neuronal differentiation. J. Neurosci. 25, 5533–5543. http://dx.doi.org/10.1523/JNEUROSCI.4883-04.2005.

Krämer, A., Green, J., Pollard, J., Tugendreich, S., 2014. Causal analysis approaches in Ingenuity Pathway Analysis. Bioinformatics 30, 523–530. http://dx.doi.org/10.1093/bioinformatics/btt703.

Kuo, Y.-M., Li, Z., Jiao, Y., Gaborit, N., Pani, A.K., Orrison, B.M., Bruneau, B.G., Giasson, B.I., Smeyne, R.J., Gershon, M.D., Nussbaum, R.L., 2010. Extensive enteric nervous system abnormalities in mice transgenic for artificial chromosomes containing Parkinson disease-associated alpha-synuclein gene mutations precede central nervous system changes. Hum. Mol. Genet. 19, 1633–1650. http://dx.doi.org/10.1093/hmg/ddq038.

Lang, D., Epstein, J.A., 2003. Sox10 and Pax3 physically interact to mediate activation of a conserved c-RET enhancer. Hum. Mol. Genet. 12, 937–945.

Le Douarin, N.M., Kalcheim, C., 1999. The Neural Crest 2nd edition.

Lommatzsch, M., Braun, a, Mannsfeldt, A., Botchkarev, V.A., Botchkareva, N.V., Paus, R., Fischer, A., Lewin, G.R., Renz, H., 1999. Abundant production of brain-derived neurotrophic factor by adult visceral epithelia. Implications for paracrine and target-derived Neurotrophic functions. Am. J. Pathol. 155, 1183–1193. http://dx.doi.org/10.1016/S0002-9440(10)65221-2.

McKeown, S.J., Wallace, A.S., Anderson, R.B., 2013. Expression and function of cell

adhesion molecules during neural crest migration. Dev. Biol. 373, 244–257. http://dx.doi.org/10.1016/j.ydbio.2012.10.028.
Miyazaki, K., Hasegawa, M., Funahashi, K., Umeda, M., 1993. A metalloproteinase inhibitor domain in Alzheimer amyloid protein precursor. Nature 362, 839–841. http://dx.doi.org/10.1038/362839a0.
Moore, M.W., Klein, R.D., Fariñas, I., Sauer, H., Armanini, M., Phillips, H., Reichardt, L. F., Ryan, A.M., Carver-Moore, K., Rosenthal, A., 1996. Renal and neuronal abnormalities in mice lacking GDNF. Nature 382, 76–79. http://dx.doi.org/10.1038/382076a0.
Natarajan, D., Marcos-Gutierrez, C., Pachnis, V., de Graaff, E., 2002. Requirement of signalling by receptor tyrosine kinase RET for the directed migration of enteric nervous system progenitor cells during mammalian embryogenesis. Development 129, 5151–5160.
Neidhart, S., Antonsson, B., Gilliéron, C., Vilbois, F., Grenningloh, G., Arkinstall, S., 2001. c-Jun N-terminal kinase-3 (JNK3)/stress-activated protein kinase-beta (SAPKbeta) binds and phosphorylates the neuronal microtubule regulator SCG10. FEBS Lett. 508, 259–264.
Ngan, E.S.W., Shum, C.K.Y., Poon, H.C., Sham, M.H., Garcia-Barcelo, M.M., Lui, V.C.H., Tam, P.K.H., 2008. Prokineticin-1 (Prok-1) works coordinately with glial cell line-derived neurotrophic factor (GDNF) to mediate proliferation and differentiation of enteric neural crest cells. Biochim. Biophys. Acta – Mol. Cell Res. 1783, 467–478. http://dx.doi.org/10.1016/j.bbamcr.2007.09.005.
Okamura, Y., Saga, Y., 2008. Notch signaling is required for the maintenance of enteric neural crest progenitors. Development 135, 3555–3565. http://dx.doi.org/10.1242/dev.022319.
Pichel, J.G., Shen, L., Sheng, H.Z., Granholm, A.C., Drago, J., Grinberg, A., Lee, E.J., Huang, S.P., Saarma, M., Hoffer, B.J., Sariola, H., Westphal, H., 1996. Defects in enteric innervation and kidney development in mice lacking GDNF. Nature 382, 73–76. http://dx.doi.org/10.1038/382073a0.
Pingault, V., Bondurand, N., Kuhlbrodt, K., Goerich, D.E., Préhu, M.O., Puliti, A., Herbarth, B., Hermans-Borgmeyer, I., Legius, E., Matthijs, G., Amiel, J., Lyonnet, S., Ceccherini, I., Romeo, G., Smith, J.C., Read, A.P., Wegner, M., Goossens, M., 1998. SOX10 mutations in patients with Waardenburg-Hirschsprung disease. Nat. Genet. 18, 171–173. http://dx.doi.org/10.1038/ng0298-171.
Plaza-Menacho, I., Burzynski, G.M., de Groot, J.W., Eggen, B.J.L., Hofstra, R.M.W., 2006. Current concepts in RET-related genetics, signaling and therapeutics. Trends Genet. 22, 627–636. http://dx.doi.org/10.1016/j.tig.2006.09.005.
Ramalho-Santos, M., Melton, D.A., McMahon, A.P., 2000. Hedgehog signals regulate multiple aspects of gastrointestinal development. Development 127, 2763–2772. http://dx.doi.org/10.1007/BF03270579.
Romeo, G., Ronchetto, P., Luo, Y., Barone, V., Seri, M., Ceccherini, I., Pasini, B., Bocciardi, R., Lerone, M., Kääriäinen, H., 1994. Point mutations affecting the tyrosine kinase domain of the RET proto-oncogene in Hirschsprung's disease. Nature 367, 377–378. http://dx.doi.org/10.1038/367377a0.
Sánchez, M.P., Silos-Santiago, I., Frisén, J., He, B., Lira, S.A., Barbacid, M., 1996. Renal agenesis and the absence of enteric neurons in mice lacking GDNF. Nature 382, 70–73. http://dx.doi.org/10.1038/382070a0.
Salehi, A., Delcroix, J.D., Belichenko, P.V., Zhan, K., Wu, C., Valletta, J.S., Takimoto-Kimura, R., Kleschevnikov, A.M., Sambamurti, K., Chung, P.P., Xia, W., Villar, A., Campbell, W. a, Kulnane, L.S., Nixon, R. a, Lamb, B.T., Epstein, C.J., Stokin, G.B., Goldstein, L.S.B., Mobley, W.C., 2006. Increased app expression in a mouse model of down's syndrome disrupts ngf transport and causes cholinergic neuron degeneration. Neuron 51, 29–42. http://dx.doi.org/10.1016/j.neuron.2006.05.022.
Sato, Y., Heuckeroth, R.O., 2008. Retinoic acid regulates murine enteric nervous system precursor proliferation, enhances neuronal precursor differentiation, and reduces neurite growth in vitro. Dev. Biol. 320, 185–198. http://dx.doi.org/10.1016/j.ydbio.2008.05.524.
Schneider, M.R., Dahlhoff, M., Horst, D., Hirschi, B., Trülzsch, K., Müller-Höcker, J., Vogelmann, R., Allgäuer, M., Gerhard, M., Steininger, S., Wolf, E., Kolligs, F.T., 2010. A key role for E-cadherin in intestinal homeostasis and paneth cell maturation. PLoS One 5, e14325. http://dx.doi.org/10.1371/journal.pone.0014325.
Schuchardt, A., D'Agati, V., Larsson-Blomberg, L., Costantini, F., Pachnis, V., 1994. Defects in the kidney and enteric nervous system of mice lacking the tyrosine kinase receptor Ret. Nature 367, 380–383. http://dx.doi.org/10.1038/367380a0.
Semar, S., Klotz, M., Letiembre, M., Van Ginneken, C., Braun, A., Jost, V., Bischof, M., Lammers, W.J., Liu, Y., Fassbender, K., Wyss-Coray, T., Kirchhoff, F., Schafer, K.H., 2013. Changes of the enteric nervous system in amyloid-beta protein precursor transgenic mice correlate with disease progression. J. Alzheimers Dis. 36, 7–20. http://dx.doi.org/10.3233/JAD-120511.
Southard-Smith, E.M., Kos, L., Pavan, W.J., 1998. Sox10 mutation disrupts neural crest development in Dom Hirschsprung mouse model. Nat. Genet. 18, 60–64. http://dx.doi.org/10.1038/ng0198-60.
Taraviras, S., Marcos-Gutierrez, C., Durbec, P., Jani, H., Grigoriou, M., Sukumaran, M., Wang, L., Hynes, M., Raisman, G., Pachnis, V., 1999. Signalling by the RET receptor tyrosine kinase and its role in the development of the mammalian enteric nervous system, vol. 2797, pp. 2785–2797.
Teng, L., Mundell, N.A., Frist, A.Y., Wang, Q., Labosky, P.A., 2008. Requirement for Foxd3 in the maintenance of neural crest progenitors. Development 135, 1615–1624. http://dx.doi.org/10.1242/dev.012179.
Tough, I.R., Forbes, S., Tolhurst, R., Ellis, M., Herzog, H., Bornstein, J.C., Cox, H.M., 2011. Endogenous peptide YY and neuropeptide Y inhibit colonic ion transport, contractility and transit differentially via Y_1 and Y_2 receptors. Br. J. Pharmacol. 164, 471–484. http://dx.doi.org/10.1111/j.1476-5381.2011.01401.x.
Trazzi, S., Fuchs, C., Valli, E., Perini, G., Bartesaghi, R., Ciani, E., 2013. The amyloid precursor protein (APP) triplicated gene impairs neuronal precursor differentiation and neurite development through two different domains in the ts65dn mouse model for down syndrome. J. Biol. Chem. 288, 20817–20829. http://dx.doi.org/10.1074/jbc.M113.451088.
Turner, P.R., O'Connor, K., Tate, W.P., Abraham, W.C., 2003. Roles of amyloid precursor protein and its fragments in regulating neural activity, plasticity and memory. Prog. Neurobiol. . http://dx.doi.org/10.1016/S0301-0082(03)00089-3
Tyan, S.-H., Shih, A.Y.-J., Walsh, J.J., Maruyama, H., Sarsoza, F., Ku, L., Eggert, S., Hof, P. R., Koo, E.H., Dickstein, D.L., 2012. Amyloid precursor protein (APP) regulates synaptic structure and function. Mol. Cell. Neurosci. 51, 43–52. http://dx.doi.org/10.1016/j.mcn.2012.07.009.
Uesaka, T., Nagashimada, M., Enomoto, H., 2015. Neuronal differentiation in schwann cell lineage underlies postnatal neurogenesis in the enteric nervous system. J. Neurosci. 35, 9879–9888. http://dx.doi.org/10.1523/JNEUROSCI.1239-15.2015.
Uesaka, T., Nagashimada, M., Yonemura, S., Enomoto, H., 2008. Diminished Ret expression compromises neuronal survival in the colon and causes intestinal aganglionosis in mice. J. Clin. Investig. 118, 1890–1898. http://dx.doi.org/10.1172/JCI34425.
Vohra, B.P.S., Tsuji, K., Nagashimada, M., Uesaka, T., Wind, D., Fu, M., Armon, J., Enomoto, H., Heuckeroth, R.O., 2006. Differential gene expression and functional analysis implicate novel mechanisms in enteric nervous system precursor migration and neuritogenesis. Dev. Biol. 298, 259–271. http://dx.doi.org/10.1016/j.ydbio.2006.06.033.
Young, H.M., Hearn, C.J., Farlie, P.G., Canty, A.J., Thomas, P.Q., Newgreen, D.F., 2001. GDNF is a chemoattractant for enteric neural cells. Dev. Biol. 229, 503–516. http://dx.doi.org/10.1006/dbio.2000.0100.
Young, H.M., Bergner, A.J., Anderson, R.B., Enomoto, H., Milbrandt, J., Newgreen, D. F., Whitington, P.M., 2004. Dynamics of neural crest-derived cell migration in the embryonic mouse gut. Dev. Biol. 270, 455–473. http://dx.doi.org/10.1016/j.ydbio.2004.03.015.
Young, H.M., Turner, K.N., Bergner, A.J., 2005. The location and phenotype of proliferating neural-crest-derived cells in the developing mouse gut. Cell Tissue Res. 320, 1–9. http://dx.doi.org/10.1007/s00441-004-1057-5.
Zizzo, M.G., Mastropaolo, M., Lentini, L., Mulè, F., Serio, R., 2011. Adenosine negatively regulates duodenal motility in mice: role of A(1) and A(2A) receptors. Bull. Int. Assoc. Eng. Geol. 164, 3–11. http://dx.doi.org/10.1111/j.1476-5381.2011.01498.x.